Neuron, Vol. 17, 979–990, November, 1996, Copyright ©1996 by Cell Press

Are Cycling Gene Products as Internal Zeitgebers No Longer the Zeitgeist of Chronobiology?

Minireview

Jeffrey C. Hall
Department of Biology
Brandeis University
Waltham, Massachusetts 02254

Oh my god, here comes another minireview about the molecular biology of rhythms. Most of these studied molecularly are low frequency, circadian ones. The highest frequency biological rhythm we have to contend with is the appearance rate of the minireviews (every 15 s). This is not just to poke fun at all of those newsy accounts. First, that is the world's easiest and most thankless task. Second, such accounts are on the verge of making dogma out of the daily molecular rhythms that are defined by oscillating abundances of clock gene products. This means four genes' worth of circadian-cycling mRNAs so far (reviewed by Hall, 1995; Sehgal et al., 1996; Dunlap, 1996). Moreover, three of the proteins encoded at these clock loci are known to exert feedback regulation of "their own" transcript cyclings. Owing to the further fact that these molecular oscillations and feedback effects are observed in both a fly and a fungus (which diverged from each other eleventy-zillion years ago), the implied pacemaker mechanism appears universal.

At least it would seem to be the case that molecular circadian rhythmicity is the spirit of the times insofar as chronobiological research is concerned. Even in species for which no clock gene candidates have been cloned, clock-controlled genes (CCGs) are well known (reviewed by Kay and Millar, 1995; Loros, 1995; Ishida, 1995). This includes cases for which mutations causing alterations of the organisms' circadian periodicities also change cycle durations of the CCGs' mRNA abundance fluctuations (e.g., Loros, 1995). In fact, some of these putative clock mutants were isolated on that very criterion (Kay and Millar, 1995; Golden et al., 1996). And a mutant phenotype that will not seem to quit (in terms of its heuristic value for identifying a clock gene at the molecular level) is one for which the circadian clock's pace is no longer "about a day," but instead some number of hours too fast or too slow. Connecting a suspected or known clock gene function to the temporally varying expression of a CCG, then, involves a short mental step: if a clock gene can control its own cycling, then why not that of other genes as well? The latter are presumed, and in some cases known, to act within output pathways that connect central pacemaking to various elements of the organism's biology (Kay and Millar, 1995; Golden et al., 1996). Thus, clock genes and their temporally regulated expression are powerful internal timegivers, being crucial contributors to the mechanisms of central pacemaking and the control of the organism's temporal order.

Varying Subcellular Localizations and Apparent Functions of Clock Gene Products

However, these transcriptionally based notions have chronically left certain observations involving clock gene expressions out in the cold. Consider the *period* (*per*) gene of Drosophila, the first one to be mutated and cloned (Konopka, 1987). *per* expression is widespread in the fly's tissues. In most tissues, PER protein is most readily observed as a nuclear signal, at least during certain portions of the daily cycle (see below). Indeed, nuclear PER and the overall level of the protein are rhythmic parameters (Rosbash et al., 1996), as is the fluctuating abundance of *per* mRNA. But PER seems relentlessly cytoplasmic in at least one tissue type, the adult ovary (Hall, 1995), and ovarian mRNA levels are temporally flat (Hardin, 1994). What is the gene doing biologically in this tissue; could the expression be merely "recreational"? (G. R. Fink, quoted in Hall, 1995). This observation cannot be dismissed, because the biological significance of *per* expression in a given body region or cell type, except for certain adult brain neurons, is unknown. Consider, however, the following little-known clock function in Drosophila: ovarian diapause, which is controlled by a circadian clock (Saunders, 1990). It is most intriguing that the 19 hr and 29 hr *per* mutations leave this pacemaker running at its normal 24 hr pace. This is not to say that the gene's function is without effect on the phenotype: a null mutation in the gene or a deletion of *per* left the photoperiodic clock running but caused the "critical day length" for the triggering of diapuse to be up to 5 hr shorter than normal (Saunders, 1990). This suggests that the odd kind of *per* expression described in the ovary is not recreational, even though it does not set the pace of the clock in this tissue.

Another piece of potential *per* heresy concerns the fact that the gene controls a high frequency rhythm in the male's courtship song; the "ultradian" cycle durations are ~1/2 min or 1 min depending on the Drosophila species (reviewed by Hall and Kyriacou, 1990). *per* exerts its influence on these singing cycles via expression in the fly's thoracic nervous system (Konopka et al., 1996). In addition to the curiosity of there being no "per^+ neurons" in these ventral ganglia, only PER-containing glia (Hall, 1995), the question arises as to how a gene product abundance rhythm could operate on a <1 min time scale. Perhaps the protein plays an altogether different kind of cellular role in these thoracic CNS cells, not necessarily a transcription-regulating one.

Interactions Between Clock Gene Products in Different Cellular Contexts

A corollary could be that PER interacts with a different array of factors in thoracic glia. This general notion is not invoked from thin air, which is thick these days with interest in the second clock gene product to be discovered in Drosophila, the TIMELESS (TIM) protein (reviewed by Sehgal et al., 1996). TIM does indeed interact with PER in the fly head (Rosbash et al., 1996). Moreover, one way TIM-encoding sequences were initially identified was in a molecular interaction screen (Sehgal et al., 1996). In addition, *tim* mRNA and TIM cycle in a circadian way, pretty much in concert with the *per* product oscillations (Sehgal et al., 1996). It is not known, however, in what posterior tissues *tim* may be expressed. Perhaps TIM cannot interact with PER in glia of the ganglia, because it is simply not expressed there. The protein interactions in the fly head have

been nicely analyzed in vivo as well as in yeast (Rosbash et al., 1996), and they seem essential for circadian cycling in that body region. For instance, where there is no TIM, there is no *per* mRNA or PER cycling, and very little PER protein at all (Price, 1995). The same PER depression occurs in wild-type heads kept in constant light (Price, 1995). Bear in mind, however, that the demonstrations of TIM/PER interactions created no information as to how these proteins participate in transcriptional control. The hypothesis that they do stems from finding that TIM influences its own mRNA cycling as well as that of *per*, and that some aspect of PER function exerts the same kind of effect on daily fluctuations of both *tim* and *per* expressions (Sehgal et al., 1996).

The only information available about the meaning of PER/TIM interactions is that the proteins' association in the cytoplasm helps mediate their entry into the nucleus; most of these data come from cultured transfected cells (Saez and Young, 1996 [this issue of *Neuron*]). More generally, the intriguingly slow process of nuclear entry (Rosbash et al., 1996) may be one reason why there is a considerable time delay between the appearance of *per* or *tim* mRNA and peak levels of the proteins in the subcellular compartment where PER and TIM would seem to carry out their principal clock-related functions. Recently, it has been found that Neurospora's *frequency* (*frq*)-encoded clock proteins (there are 2 translational products) also peak a few hours after the *frq* mRNA does (Dunlap, 1996). All of this allows one to understand as a first approximation why the PER, TIM, and FRQ feedback effects are negative: as the proteins are going up, the RNAs go down. An additional aspect of changeable TIM levels is that clock-resetting light stimuli cause a rather abrupt decrease in the protein's abundance (reviewed by Sehgal et al., 1996). It is different for FRQ, but analogous: light makes *frq* mRNA go up (Dunlap, 1996), and this species difference jibes with the *frq* transcript peak being about a half-day out of phase with those that define *per* or *tim* mRNA cyclings. How these light effects on the oscillating gene products would mediate the phase shifts (delays early in the night; advances during the second half of it) are readily rationalized (discussed by Sehgal et al., 1996; Rosbash et al., 1996; Dunlap, 1996).

Relatives of Drosophila's Clock Genes in Other Species: Expression Pattern Surprises and Puzzles

But a pair of papers in this issue of *Neuron* seem to have stuck a moth in the ointment. These experiments represent a maturing story that started with cloning the first bona fide *per* homolog in a species outside the Drosophila genus (Reppert et al., 1994). A silkmoth was chosen in part because Steven M. Reppert has long been interested in those insects irrespective of them being research objects; coincidentally, so has the fellow who started this entire cottage industry by deliberating searching for and finding the first *per* mutant in the summer of '68 (Konopka, 1987). There were other a priori reasons to move molecular studies of insect rhythms sideways: information about cells and substances related to silkmoth rhythms in particular is much more extensive than in Drosophila, as is the accessibility and manipulability of the pertinent tissues (e.g., Truman et al., 1993).

But before discussing why cloning *per* from the giant silkmoth, Antheraea pernyi, had its own reward, a few pieces of additional history: the interspecific comparative approach to molecular chronobiology started not long after *per* was first cloned in Drosophila (reviewed by Ishida, 1995). Yet no real *per* homologs were findable in distant organisms such as vertebrates, perhaps because *per* is one of the most divergent genes among Drosophila species (reviewed by Kyriacou et al., 1996). However, some intragenic regions are quite conserved, and this holds up when one compares the various fly *per*'s to the silkmoth one (Reppert et al., 1994). Furthermore, antibodies made against a subset of perhaps the most evolutonarily conserved subset of PER lead to staining of known clock structures in a variety of other organisms (e.g., Siwicki et al., 1989; Rosewell et al., 1994). The problems with these studies included a lack of cloning-based identification of the antigens (are they truly *per* relatives?), and that the antibodies stained non-clock structures in the nervous systems of these other invertebrates and even some vertebrates (Rosewell et al., 1994). However, recall that genuine *per* expression in Drosophila is all over the place (Hall, 1995). One of the most problematic features of the interspecific investigations is that the intracellular signals in the organisms examined for "PER-like" immunoreactivity at highest resolution are not nuclear and mark the entire lengths of the processes projecting from neuronal cell bodies whose cytoplasms stain (Siwicki et al., 1989).

Similar results are found in the current papers: anti-PER-mediated stainings in the CNS and visual system of a beetle are in the main non-nuclear and neurite-filling (Frisch et al., 1996). Even though the antibody seems to label several elements of this non-dipteran insect's circadian system, one downplays the significance of these stainings and of the analogous ones in molluscs: PER, and now TIM, are supposed to be doing their business in the nuclei of the relevant fly neurons, so the beetle and Bulla neurons, for example, would be stained because of cross-reacting material that is uninteresting in terms of molecular chronobiological mechanisms.

Now one wonders if these occult cytoplasmic signals may be real after all. In the silkmoth, Reppert, Ivo Sauman, and colleagues report some heretical PER and TIM expression patterns of their own, that is, of the silkmoth's CNS neurons. Antibodies against the same intra-PER region as mentioned above showed a very small number of *per* neurons in the silkmoth brain (Sauman and Reppert, 1996 [this issue of *Neuron*]). The signal was restricted to the perikaryal cytoplasm and to the axons of those cells. These were shown to be bona fide *per* cells by colocalization of the mRNA in situ (Sauman and Reppert, 1996). This was not possible in the more primitive interspecific forays discussed above. Another difference is that the silkmoth *per* products oscillate in the brain neurons (the overall immunochemical evidence on this point for the other non-fly organisms is equivocal, although see Rosewell et al., 1994). The current investigators deepened their analysis of clock gene expressions in the silkmoth's adult brain in several ways (Sauman and Reppert, 1996): first, the *per* mRNA peaks or troughs were found not to "phase-lead" those for PER protein by any appreciable number of hours; second, *tim* expression, inferred from application of anti-fly TIM, was shown to colocalize with that of *per* (TIM-like material cycled as a cytoplasmic signal in phase with the

Table 1. Varying Expression Patterns and Functional Meanings of Clock Genes

Function	FRUITFLY					SILKMOTH			
	Brain		Eye	Gut	Ovary	Brain		Eye	Gut
	Emb.	Ad.				Emb.	Ad.		
Nuclear	?	Yes	Yes	Yes	No	No	No	Yes	Yes
Cycling	?	Yes	Yes	Yes	No	No	Yes	Yes	Yes
Biological Meaning	?	Yes	?	?	Yes	Yes	?	?	?
Pacemaking Function	?	Yes	?	?	No	?	?	?	?

The data supporting this summary of descriptions of clock gene product expression patterns and manipulations of them to determine the chronobiological roles played by the *per* and, to a more limited extent, *tim* can be found in or reviewed by Saunders (1990), Hardin (1994), Reppert et al. (1994), Hall (1995), Sehgal et al. (1996), Rosbash et al. (1996), Sauman and Reppert (1996), and Sauman et al. (1996). The generic noun Gut refers to essentially the entire alimentary canal of the Drosophila adult, and to the mid-gut epithelium of embryonic A. pernyi. With respect to the time- and light-related ovarian phenotype in Drosophila, it is not yet known if *per* expression in that tissue is autonomously connected with the effects of certain *per* mutations on one diapause parameter. Abbreviations: Emb., embryo; Ad., adult.

PER fluctuation); third, constant light abolished all of these gene product rhythms (as it is known to eliminate circadian biological rhythms in silkmoth as well as in Drosophila); and fourth, expression of in vivo antisense *per* RNA (!) was discovered within these same silkmoth brain neurons; morever, the noncoding *per* transcript oscillates in anti-phase to the coding one.

Trying to Interpret Clock Gene Expression Patterns in Different Organismic and Cellular Contexts

Before wondering what this may mean mechanistically, does it mean anything biologically? For this, Reppert, Sauman, and coworkers turned to a manipulable rhythm earlier in the life cycle: temporally gated egg-hatching. By injecting exogenous antisense *per* RNA, the circadian hatching rhythm was eliminated (Sauman et al., 1996 [this issue of *Neuron*]). Assessments of expression accompanying this experiment showed that antisense injections into embryos effected a substantial decrease in PER immunoreactivity. In controls, PER and TIM were observed in a small number of embryonic brain neurons; this staining was not only restricted to the cytoplasm (as in adults), but also did not cycle, whereas nuclear PER cycling was observed in the developing gut (Sauman et al., 1996). Analogous cyclical signals are observed in the gut of Drosophila adults (Table 1). *per* RNA in the silkmoth (sense only) was examined for cycling in whole embryo extracts; the signal was flat (Sauman et al., 1996; Table 1). But the antisense effects on the hatching rhythms indicate that these investigators are dealing with functionally meaningful *per* in this insect.

However, the silkmoth is still far from having the function of its postembryonic neuronal pacemakers manipulated in situ. Thus, whereas the *per/tim* neurons in the silkmoth's brain probably are involved in behavioral rhythms of the adult (Truman et al., 1993), this part of the life cycle is not yet accessible to mutational and molecular manipulation. Some dark night, however, someone will transform in vitro mutagenized versions of *per* and *tim* into A. pernyi (cf. Ashburner, 1995). Even in a normal genetic background, such transgenes are quite likely to cause chronobiological abnormalities, owing to the relentless semidominance of clock mutations (Hall, 1995; Dunlap, 1996). In particular, the alterations in adult behavior that could result from the silkmoth mimic of a per^{short} or per^{long} mutation might be a more definitive test of function than knocking out the hatching rhythms by bringing down embryonic PER. That silkmoth PER can set the pace of the clock in adult Drosophila (Levine et al., 1995) does not mean that it normally functions in this manner in moth brain. This concern is more than a matter of i-dotting and t-crossing, owing to the strikingly different interspecific intracellular expression patterns (Levine et al., 1995; Sauman and Reppert, 1996).

There are some intriguing similarities for the clock gene expression patterns in the silkmoth and the more manipulable fly: the initial report of the *per* gene cloned from A. pernyi included the demonstration that mRNA taken from the whole head cycled, as did immunohistochemically detected PER in photoreceptors of the compound eye (Reppert et al., 1994). The molecular picture for the eye now comes into sharper focus since PER signals within the photoreceptors are distinctly nuclear (Reppert et al., 1994) and the *per* mRNA in this tissue cycles out of phase with that of PER (Sauman and Reppert, 1996). The peak for the former leads that of the latter by a few hours, as is the case for all known cell types that express the two clock genes in question in the Drosophila head (Table 1).

It follows from the qualities of the molecular rhythms running in these insect photoreceptors that the mechanisms of *per* (and *tim*) function may be the same, that is, within particular tissues of silkmoth and fruitfly. What about the cytoplasmic clock proteins and the *per* antisense mRNA in the silkmoth's brain? One can only speculate about the functional significance of these molecularly surprising expression patterns. For instance, if the in vivo antisense mRNA in adult neurons helps bring PER down at the appropriate trough time, the cytoplasmic nature of that protein still is mysterious. (Neurospora's *frq* clock gene also generates an antisense RNA, incidentally; Dunlap, 1996). The notion that PER in the silkmoth could function in some sort of neurohumoral manner—being a potentially secreted protein, because it goes down to the end of the axons (Sauman and Reppert, 1996)—is a reach.

One is left with the softly stated possibility that the products of rhythm-regulating genes in certain chronobiologically relevant cells act instead as accessory proteins. They could be concerned, for example, with moving information into the cell or between subcellular compartments. Within one such location, the nucleus of any pacemaker cell, it could still be the case that

other workhorse molecules are setting the actual pace of the circadian clock. These notions lead to the specific suggestion that silkmoth PER in the brain (but not within photoreceptors or the embryonic gut), and fly PER in the ovarian follicle cells (but not the brain), play an accessory role, and that the workhorses just mentioned are yet to be discovered. The general implication is that there is context dependence for the functional meaning of a given molecule. This refers to more than descriptions of where such a molecule is found with cells and whether its concentration defines a daily rhythm (see Table 1 for a summary). In addition, fly PER's functional significance seems to be rather different in the ovary than in brain neurons (Saunders, 1990), if it is the case that the critical day length shortening is due to an autonomous effect of *per* mutations in the ovary (not known). Also, the context in which silkmoth PER finds itself can make a substantial difference, given that this material sets the pace of the clock and is a nuclear protein when the A. pernyi gene is transformed into flies (Levine et al., 1995). It is gingerly suggested that if mutated forms of A. pernyi *per* do make it into transgenic silkmoths, only marginal effects on the adult behavioral rhythms will result by analogy to Saunder's genetic studies of ovarian diapause. If that result is found, one will reflect back on the matter of knocking out the silkmoth hatching rhythm (by the dominant-negative effect discussed above); in retrospect, this would be viewed as falling short of indicating that this insect's *per* gene functions as a "state variable," a part of the machinery that actually runs the clock at its 24 hr pace (discussed by Kay and Millar, 1995; Dunlap, 1996). In other words, the hatching arrhythmicity might in a sense be a marginal phenotypic abnormality. "Marginal" is not meant to be pejorative; the silkmoth PER product could be a required part of the molecular output pathway that connects central pacemaking to temporally gated hatching; this suggestion was indirectly made in the companion paper (Sauman and Reppert, 1996).

It seems that much more needs to be done (although not this month), with respect to molecular rhythmicity in A. pernyi. For example, consider one of the criteria that core features of circadian oscillators should fulfill: abrupt changes in the level of a clock component should cause stable phase shifts in the overt biological rhythm. That could be a straightforward study in silkmoth. One does these experiments by delivering clock-resetting light pulses to wild-type versions of the organism—so far, flies or fungi (reviewed by Rosbash et al., 1996; Dunlap, 1996). Therefore, establishment of the molecular probes for silkmoth clock gene products (Reppert et al., 1994; Sauman and Reppert, 1996) has quickly put this system in a position to ask whether these products are state variables for clock operation. And since the mass of this insect approaches that of a beached whale, separate assessments of light pulse effects on *per* and *tim* product levels in different tissues seem within reach for near term investigations.

It already seems almost certain that homologs of the *per* and *tim* genes in A. pernyi are participating in the regulation of that organism's rhythms. However, from an older perspective, one that was stuck in flies for many years, some chronobiologists have been groping for ways to appreciate the overall mechanistic meaning of putative clock gene products. They have been unable to get around the fact that at least a fraction of such molecules seem to act pleiotropically, based at least on the effects of the mutations (this includes ultradian rhythm abnormalities in a mammalian circadian rhythm mutant: Loudon et al., 1994). Or the gene products might act in this manner, as inferred from descriptions of intraorganismal variations in cellular expression patterns. The current silkmoth papers reinforce the suspicion that one attribute of these genes is versatility of function at the level of cellular biochemistry—mechanistic pleiotropy, if you will. To learn whether the scope of these gene actions will continue to expand, or whether more unifying principles will ultimately emerge, we eagerly await the cloning of genes defined by rhythm mutations in the non-insect organisms referred to near the beginning of this minireview. The publications that prompted the review (Sauman and Reppert, 1996; Sauman et al., 1996) reveal forcefully how provocative the interspecific comparative approach can be.

Selected Reading

Ashburner, M. (1995). Science *270*, 1941–1942.

Dunlap, J.C. (1996). Annu. Rev. Genet. *30*, 579–601.

Frisch, B., Fleissner, G., Fleissner, G., Brandes, C., and Hall, J.C. (1996). Cell. Tissue Res., in press.

Golden, S.S., Ishiura, M., Johnson, C.H., and Kondo, T. (1996). Annu. Rev. Plant Physiol. Mol. Biol., in press.

Hall, J.C. (1995). Trends Neurosci. *18*, 230–240.

Hall, J.C., and Kyriacou, C.P. (1990). Adv. Insect Physiol. *22*, 221–298.

Hardin, P.E. (1994). Mol. Cell. Biol. *14*, 7211–7218.

Ishida, N. (1995). Neurosci. Res. *23*, 232–240.

Kay, S.A., and Millar, A.J. (1995). Cell *83*, 361–364.

Konopka, R.J. (1987). Annu. Rev. Genet. *21*, 227–236.

Konopka, R.J., Kyriacou, C.P., and Hall, J.C. (1996). J. Neurogenet., in press.

Kyriacou, C.P., Sawyer, L.A., Piccin, A., Couchman, M.E., and Chalmers, D. (1996). Semin. Cell Dev. Biol., in press.

Levine, J.D., Sauman, I., Imbalzano, M., Reppert, S.M., and Jackson, F.R. (1995). Neuron *15*, 147–157.

Loros, J. (1995). Semin. Neurosci. *7*, 313.

Loudon, A.S.I., Wayne, N.L., Krieg, R., Iranmanesh, A., Veldius, J.D. (1994). Endocrinology *235*, 712–718.

Price, J.L. (1995). BioEssays *17*, 583–586.

Reppert, S.M., Tsai, T., Roca, A.L., and Sauman, I. (1994). Neuron *13*, 1167–1176.

Rosbash, M., Allada, R., Dembinska, M.E., Guo, W.Q., Le, M., Marrus, S.B., Qian, Z., Rutila, J.E., Yaglom, J., and Zeng, H. (1996). Cold Spring Harbor Symp. Quant. Biol. *61*, in press.

Rosewell, K.L., Siwicki, K.K., and Wise, P.M. (1994). Brain Res. *659*, 231–236.

Saez, L., and Young, M.W. (1996). Neuron *17*, this issue.

Sauman, I., and Reppert, S.M. (1996). Neuron *17*, this issue.

Sauman, I., Tsai, T., Roca, A.L., and Reppert, S.M. (1996). Neuron *17*, this issue.

Saunders, D.S. (1990). J. Biol. Rhythms *5*, 315–331.

Sehgal, A., Ousley, A., and Hunter-Ensor, M. (1996). Mol. Cell. Neurosci. *7*, 165–172.

Siwicki, K.K., Strack, S., Rosbash, M., Hall, J.C., and Jacklet, J.W. (1989). Neuron *3*, 51–58.

Truman, J.W., Hewes, B., and Ewer, J. (1993). In Insect Neurochemistry and Neurophysiology, A.B. Borkovec and M.J. Loeb, eds. (Boca Raton, Florida: CRC Press), pp. 39–51.